tion of PGA but also competes with the product (CF) derived from PGA. The lethal action in rats of aminopterin, 25 μg daily, is not prevented by PGA, but the daily administration of 250,000 units of CF completely counteracts the toxic effects of the antagonist. That CF is a biologically active derivative of PGA is shown by the fact that rats in which growth is arrested by PGA-deficiency, and which due to aminopterin are refractory to PGA, grow remarkably when given concentrates of the citrovorum factor.

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Tocopherol Requirements of Rats by Means of the Hemolysis Test.* (17923)

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It has been found that in rats deficient in vitamin E, injection of alloxan or its reduction products, alloxatin and dialuric acid, is followed within a few minutes by hemolysis so severe that in some cases the cell volume is reduced from the normal value of over 50% to 10% or less(1,2). The same reaction may be produced in washed red blood cells with dialuric acid(2,3). Alloxan itself is not hemolytic *in vitro* so it may be presumed that its action *in vivo* depends on reduction to dialuric acid by sulfhydril or other reducing compounds. *In vivo* or *in vitro* the blood cells of animals receiving adequate amounts of tocopherol are completely protected against the hemolyzing effect of dialuric acid. The *in vitro* hemolysis test requires only a few drops of blood and has proved to be a convenient method of studying some aspects of the requirement and utilization of tocopherol in the rat.

Experimental. The rats used in these studies were females of the Sprague-Dawley strain weighing about 70 g when they were received. Depletion studies were not begun until the rats weighed about 110 g. Until this time they were given the Steenbock stock diet(4) supplemented with milk and cabbage, and the animals referred to as "stock" or "normal" received this ration throughout the whole experimental period. The vitamin E-deficient diet contained casein (General Biochemicals, vitamin test), 20; lard, 38; cod liver oil, 2; salt mixture (U.S.P. No. 2), 4; sugar, 36; and in some instances yeast, 5 (replacing an equal weight of sugar). Each animal received a daily supplement of 20γ thiamine chloride, 100γ calcium pantothenate, 20γ pyridoxine, and 25γ riboflavin. In most of the experiments the vitamin E supplement was given in the form of mixed natural tocopherols (Distillation Products) dissolved in cottonseed oil (when massive doses were given) or in peanut oil. In one study a synthetic DL-alpha tocopherol (Merck) was used.

For the hemolysis test 8 or 10 drops of blood

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were collected from the tail into 1 or 2 ml of a solution containing 0.9% of sodium chloride and 1% of sodium citrate in a graduated centrifuge tube. The blood was centrifuged, the supernatant fluid removed and 0.9% sodium chloride added to make a 5% suspension of the blood cells. To 0.25 ml of this suspension in a small tube were added 0.18 ml of phosphate buffer (25 ml of 0.2 M potassium monophosphate, 19.7 ml of 0.2 M sodium hydroxide, with water to make 100 ml) and 0.07 ml of a 0.1% solution of dialuric acid in the buffer. The tubes were incubated for 15 min. at 37°C. after which they were kept at room temperature and the time required for the cell suspension to become clear noted. A control tube without dialuric acid was run with the test sample. When the blood cells of animals which had received the deficient diet for several weeks were tested, there was complete hemolysis within half an hour. If the cells were partially protected either because of a shorter depletion period or an inadequate intake of tocopherol, hemolysis occurred, but more slowly. In the following discussion hemolysis (or tocopherol deficiency) has been classified as slight if 4 or more hours were required for the suspension of red blood cells to become completely clear, moderate if there was complete lysis in 1 to 4 hours. The blood of a tocopherol-treated animal was run as a control with each test. No hemolysis was ever observed in these cells.

Results. Rate of tocopherol depletion. The red blood cells of animals receiving the stock ration usually showed no hemolysis when treated with dialuric acid. In a few cases a faint trace was observed. After these rats had received the tocopherol-deficient diet for only 3 days there was definite, though slight, hemolysis; in 7 days it had increased considerably, and after 10 days almost the maximum rate of hemolysis had been reached. In marked contrast to the rapid tocopherol depletion in these animals was the response of a group which, for a considerable period, had been given 3 mg of tocopherol daily as a supplement to the deficient diet, and then kept on the deficient diet alone. Four of these rats which had received the supplement for about 100 days showed only slight hemolysis after 118 days without tocopherol. Two animals which had received tocopherol for 44 days showed only this same slight sensitivity to dialuric acid after deprivation for 91 days. The tendency to hemolysis increased very slowly after this time. Three animals died before they were completely depleted. Nine months after treatment was discontinued hemolysis had reached the maximum rate in two of the remaining animals while the blood of the third was still partially protected.

TABLE I.
Effect of Tocopherol Administration to Rats on the Sensitivity of the Red Blood Cells to Hemolysis by Dialuric Acid. (10 rats in each group).

DL-alpha tocopherol, mg/day	No. of rats protected: Time after addition of dialuric acid to R.B.C. 1 hr	2 hr	4 hr
.00	0		
.10	2	0	
.14	4	1	0
.20	8	4	0
.28	10	10	8

Protective and curative doses of tocopherol. To determine how much tocopherol was required to protect the red cells, rats were transferred to the vitamin E-deficient ration and groups of 2 given 0.05, 0.10, 0.20, and 0.40 mg of mixed natural tocopherols daily. When the animals had received the experimental ration for 2 weeks the blood was tested and only with the highest dose of tocopherol was hemolysis completely prevented. In a more extended study, groups of 10 animals were given 0.10, 0.14, 0.20 and 0.28 mg per day of DL-alpha tocopherol. The results are shown in Table I. There is good proportionality between the dose and the number of rats protected. The highest dose given was a little less than optimal since 2 animals were not completely protected. If partial rather than complete protection of the red cells had been tabulated, a somewhat greater protective effect of the lower doses of tocopherol would have been demonstrated. In the control group the cells of every rat were completely hemolyzed in less than one hour. Of the animals receiving 0.10 mg of tocopherol

only 2 showed complete hemolysis in one hour and with the higher doses, in no animal was there more than partial hemolysis in this period. The deficiency of tocopherol in the erythrocytes could be rapidly overcome. Two rats which had received the deficient ration for one month were given a single dose of 1.5 mg of natural tocopherols. The red cells were completely resistant to hemolysis the next day, and some protective effect persisted for about 10 days. A dose of 0.5 mg was inadequate for demonstration of any protective effect in 24 hours. When the dose was repeated for 3 days there was some protection but it was never complete and disappeared as soon as therapy was discontinued.

Tocopherol in the blood of newborn rats. Transfer of tocopherol across the placenta is known to be poor, and it was of interest to determine the response of the erythrocytes of newborn rats to dialuric acid. Sufficient blood for a determination could be obtained from 1 or 2 animals by decapitation. Litters of 4 females receiving the stock ration were studied. Two of these litters were born during the day and the blood of the young rats was tested within an hour. Hemolysis was complete in less than an hour. Hemolysis was slower in 2 litters born during the night and tested the following morning, but a moderate degree of tocopherol deficiency was still indicated. Three of the litters were completely protected against hemolysis on the second day of life while the blood of the rats in the fourth litter was still sensitive to dialuric acid. The animals of this litter were in poor condition and probably had received no nourishment. Four females were given 5 mg a day of tocopherol throughout the gestation period. There was no hemolysis in any of the young tested. Unfortunately, all of the litters were born at night and the possibility that they might have been deficient in tocopherol at birth was not entirely precluded. In order to make sure that the difference between the 2 groups was not due to any post-natal influences, caesarian section was performed at about the twentieth day of pregnancy on 2 females receiving the supplement of tocopherol and 2 females given the unsupplemented diet. There was no hemolysis in the fetuses of the first group, while in the second, hemolysis was observed as with the newborn litters.

Plasma tocopherol determinations. In order to compare the degree of tocopherol deficiency measured by susceptibility to hemolysis with deficiencies described in the literature, tocopherol was determined in the plasma of stock, tocopherol - deficient and tocopherol - treated animals. Some of the deficient animals and all of the tocopherol-treated animals had received an injection of alloxan or dialuric acid one or 2 months previously, from which they had recovered without serious after-effects. The method of Hines and Mattill(5) utilizing the colorimetric procedure devised by Emmerie and Engel(6) was used. A simple, one-step extraction which Quaife and Harris (7) found satisfactory for plasma was substituted for the more laborious procedure necessary with muscle and liver. The tocopherol-treated animals received 3 mg of tocopherol a day as a supplement to the deficient diet for about 2 months and then were returned to the stock diet and given a single dose of 9 mg of tocopherol once a week. The average value of plasma tocopherol in this group was of the same order as the normal human value reported by a number of workers: 1.0 ± 0.08 mg per 100 ml. The plasma of the rats receiving the stock ration contained less than half this amount of tocopherol, 0.38 ± 0.02 mg per 100 ml while the level in the group on the deficient diet, 0.27 ± 0.04 mg per 100 ml was not a great deal lower than that of the stock animals.

Discussion. Susceptibility to hemolysis by dialuric acid differs from other manifestations of vitamin E deficiency in the rat in the short time required for its demonstration in relatively mature animals. Signs of deficiency in the blood cells could be detected in rats 2 to 3 months old after only a few days on the tocopherol-deficient diet, and the

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maximum rate of hemolysis was reached within 2 weeks. Approximately 3 mg of DL-alpha tocopherol per kg of body weight per day was required to protect the red cells. This is several times the average minimum prophylactic dose for prevention of sterility or muscular dystrophy(8).

In comparing the animals receiving the stock ration with those given supplements of tocopherol or a tocopherol-deficient diet, it is disturbing to see how close these presumably normal animals were to the deficient level. This is apparent not only from the plasma tocopherol values but from the depletion studies. During the period of treatment rats given supplements of tocopherol stored an amount of it sufficient to maintain them for 6 to 9 months above the deficient level as measured by susceptibility to hemolysis. The normal animals were young when they were put on experiment and one would not expect a large reserve of tocopherol. The fact that they were depleted within a week indicated that there was practically no reserve. A similar observation was made by Hines and Mattill(5).

There have been a number of reports in the literature as to the relative deficiency of the newborn in tocopherol(9,10,11). If, as our experiments have suggested, tocopherol serves as a protective agent against damage to erythrocytes (and perhaps other tissues) by toxic substances, the fetus is in a peculiarly defenseless position, and may be damaged by conditions which would not affect the mother.

In our experiments the young rats were completely protected against the hemolyzing effect of dialuric acid within one day. Mason (9) observed that in sucklings 24 to 48 hours old the tocopherol content was increased to the adult level. It has been found in cows(12) and ewes(13) as well as in human beings(14) that colostrum contains several times as much tocopherol as late milk. The tocopherol deficiency of the red blood cells of the infant rats could be prevented by supplementing the ration of the mother with tocopherol during the gestation period. Whiting and Loosli(13) reported similarly that the tocopherol content of the blood plasma of newborn sheep and goats was increased by prepartum supplementation with tocopherol. Parrish, *et al.*(15) on the contrary, found little effect of prepartum supplementation of the dams with tocopherol on the plasma tocopherol level in calves.

Of the various manifestations of vitamin E deficiency, fetal resorption has proved most satisfactory for bioassay. However, the method is cumbersome and results from different laboratories are not entirely consistent. The studies of the treatment and prevention of tocopherol deficiency which have been described indicate that the change in the red blood cell may be utilized in the assay of vitamin E. The procedure has the advantages of speed and simplicity. Whether it has the necessary precision will be determined by further tests.

Summary. The sensitivity of the red blood cells of tocopherol-deficient rats to the hemolyzing action of dialuric acid *in vitro* has been applied to the study of tocopherol requirements. Rats which had received the stock diet until they were 2 to 3 months old showed signs of deficiency after only a few days on a tocopherol-deficient diet. Animals which had received large supplements of tocopherol were protected many months after it was discontinued. About 3 mg of DL-α-tocopherol per day per kg of body weight was required for protection of the erythrocytes. The newborn young of rats which had received the stock ration were susceptible to the hemolyzing action of dialuric acid. If the

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females were given a supplement of tocopherol throughout the gestation period the blood cells of the young rats were protected. The possible application of this hemolysis method to the bioassay of vitamin E has been discussed.

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Experimental Control of Serum Calcium Levels *in Vivo*.* (17924)

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The constancy of the total calcium level of the blood is the result of the interplay of numerous factors which include calcium intake, absorption and excretion, Vitamin D economy, parathyroid gland function, and blood phosphate, bicarbonate, protein and pH levels(1). The relations which determine the distribution of the total blood calcium between the ionic and the bound forms are largely unknown. It is generally accepted that the ionic calcium in the blood is the physiologically active form. Were it possible to control the ionic blood-calcium levels by exogenous means further insight into the relationships and mechanisms which govern calcium levels and distribution might be obtained. Soluble oxalates lower blood calcium ions by precipitation of insoluble calcium oxalate. Citrates, tartrates, phthalates, fluorides, polyphosphates, glycine, and other anions lower blood calcium ion by the formation of soluble nonionic complexes(2). The characteristic physiological sequelae attributable to hypocalcemia follow the administration of these agents. However, the use of these materials for quantitative control of calcium ion *in vivo* is subject to certain experimental difficulties. Ethylenediamine tetra-acetic acid (E.D.T.A.A.) forms soluble nonionized complexes with many bivalent cations(3) and behaves as a chelating agent. Dyckerhoff *et al.*(4) have reported that E.D.T.A.A. inhibits blood coagulation *in vitro* probably as the result of the removal of calcium ion by complex formation. At physiological pH we have found that in human or rabbit serum the complex of calcium ion with E.D.T.A.A. forms stoichiometrically. Calcium ion bound in E.D.T.A.A. complex is not precipitated by the addition of oxalates and hence the total remaining non-complexed calcium which may consist of both ionic and protein bound calcium can be determined by oxalate precipitation. We have demonstrated this by numerous *in vitro* experiments. That the same combination occurs *in vivo* is illustrated in Fig. 1 where the physiological action of E.D.T.A.A. was shown to be the consequence of its combination with calcium ion. Rapid (20 sec.) intravenous injection into the marginal ear vein of rabbits of E.D.T.A.A. as the neutral sodium salt, 100 mg/kg, resulted in immediate lowering of the available calcium level with consequent death by hypocalcemic tetany. When the calcium chelation of E.D.T.A.A. has been saturated by admixture with stoichiometric quantities of calcium in the form of its chloride or gluconogluconate salt prior to injection, the previously demonstrated toxic effects did not occur (Fig. 1). A second injection of the neutral sodium salt of E.D.T.A.A. 100 mg/kg, then caused

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